The uropygiols: identification of the unsaponifiable constituent of a diester wax from chicken preen glands

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ABSTRACT The chief lipid fraction in the uropygial gland excretion of the domestic hen is a diester wax. The saponifiable fraction of this wax consists of saturated normal C_{10}–C_{20} fatty acids. The unsaponifiable fraction consists of a series of three homologous compounds, which have been named the uropygiols and identified by mass spectrometry, gas–liquid chromatography, and periodate cleavage as 2,3-*n*-alkanediols containing 22–24 carbon atoms. The native diols were shown to consist of about equal amounts of the *threo* and *erythro* isomers. Records of analyses of the natural products as well as related synthetic compounds are shown.

KEY WORDS uropygiols · uropygial gland · hen · diester waxes · 2,3-*n*-alkanediols · acetonides · gas–liquid chromatography · mass spectrometry

THE UROPYGIAL (preen) gland of the chicken (Order: Galliformes) contains a substance resembling a glyceride but separable from the latter by TLC. In a preliminary note (1) we reported that saponification of this lipid yields an acid fraction containing C_{10} to C_{20} fatty acids. The chromatogram of these acids is shown in Fig. 1; it is clear that they differ considerably from the branched-chain fatty acids found in domestic geese and ducks (Order: Anseriformes) by Odham (reference 2 and preceding papers). In the same note (1) we reported that the unsaponifiable fraction from these birds consisted of a waxy solid with a TLC mobility similar to that of 12-hydroxyoctadecanol. The present report describes the final identification of this novel lipid constituent.

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-laver chromatography.

MATERIALS AND METHODS

Isolation of the Natural Diester Wax from Chicken Uropygial Gland

The uropygial glands of 10 chickens, 6–8 wk old, were isolated within 2 hr after sacrifice. The contents of the glands were squeezed directly into 100 ml of methanol–chloroform 1:1. The suspension was then brought to boiling and filtered, and the solvents were evaporated in a rotating evaporator at 40°C. The residue was extracted three times with about 5 ml of hexane, and the extracts were applied directly to a 15 × 300 mm silicic acid chromatography column. The column was then eluted with 250 ml each of benzene–hexane 2:98, benzene–hexane 20:80, and chloroform.

The desired lipid was found in the second eluate and its yield by the above procedure was 500 mg. Small portions of this sample were purified for further analyses by means of preparative TLC in benzene–hexane 7:3 on Silica Gel G.

Isolation of the Saponifiable and Unsaponifiable Fractions of the Purified Diester Wax

310 mg of the lipid was subjected to alkaline hydrolysis in potassium hydroxide–methanol–toluene as described by Nikkari (3) for other lipids. The saponifiable and unsaponifiable fractions were extracted and subsequently purified and isolated by Florisil column chromatography (3), with solvent volumes and column dimensions increased to about 10 times those described in the original paper. The fatty acids were converted to methyl esters with 2% sulfuric acid in methanol. For final analyses the methyl esters as well as the unsaponifiable fraction were purified by preparative TLC with benzene and

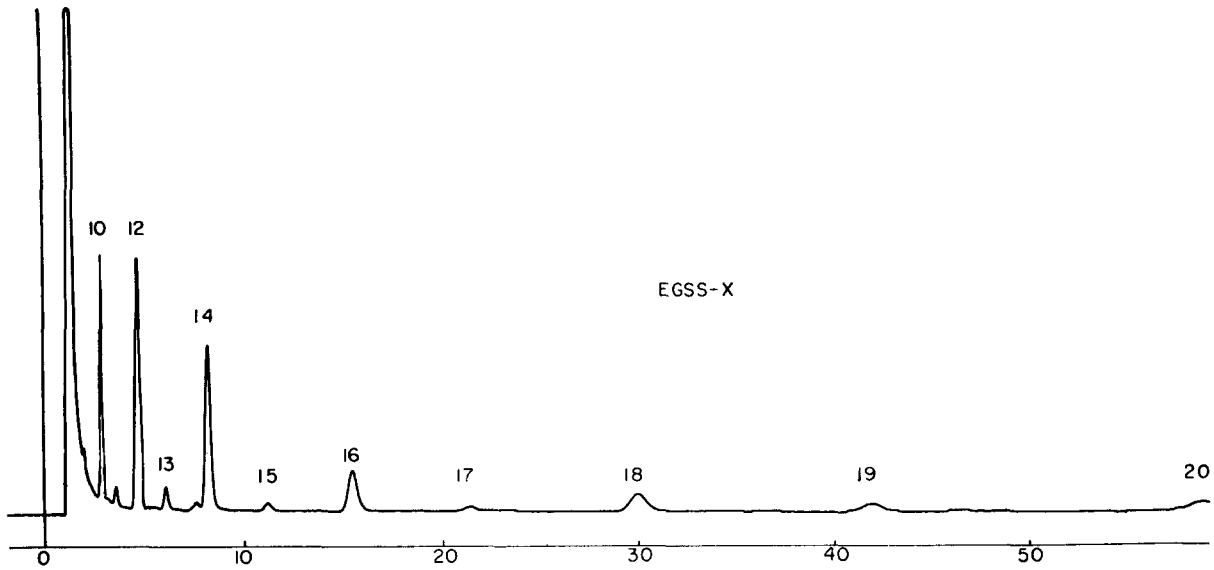


FIG. 1. Gas chromatogram of the methyl esters of fatty acids esterified with the uropygiols. Column (B): 3% ethylene glycol succinate polyester combined with a silicone (EGSS-X). See Methods for other columns employed.

ethyl acetate–chloroform 1:4 as solvents respectively. Portions of 1–5 mg of the purified uropygiols were acetylated with acetic anhydride–pyridine 1:1. The acetylated compounds were purified by preparative TLC on Silica Gel G with benzene as solvent.

Preparation of 1,2-Nonadecanediol and 2,3-Nonadecanediol from 1-Nonadecene and 2-Nonadecene Respectively

The commercial hydrocarbons (Chemical Procurement Laboratories, Inc., N. Y.) were purified by preparative GLC as were the acetonides (see below). The 1,2-nonadecene was found to contain a small amount of lower homologue which was removed in this process. The 2,3-nonadecene contained several homologues and positional isomers, including 1,2-nonadecene. The major fraction was collected even though it was composed of at least two very close peaks (GLC). On hydrogenation this fraction gave only one peak which corresponded to *n*-nonadecane. Subsequent oxidation (see below) showed that the fraction collected consisted of about 90% of an equal mixture of *cis*- and *trans*-2,3-nonadecenes while most of the remainder was 1,2-nonadecene.

The purified olefins were treated with osmium tetroxide as follows. About 20 mg of purified nonadecene was added to an equal weight of OsO_4 in 20 ml of hexane. A brown precipitate formed almost immediately. The mixture was allowed to stand overnight and then an equal quantity of 50% ethanol was added together with 2 g of sodium sulfite, and the mixture was refluxed for 4 hr. The hexane layer was then extracted and evaporated, and the diol was recrystallized from aqueous ethanol and sublimed at 120°C (1 μ).

1,2-Nonadecanediol, mp 80–82°C.

Threo- and erythro-2,3-nonadecanediols, mp 75–83°C.

Preparation of the Acetonides of the Uropygiols and Synthetic Diols

Portions of 1–2 mg of the diols were dissolved in 1 ml of acetone and 1 mg of toluene *p*-sulfonic acid was added. The mixture was left at room temperature for 10–20 min. The tube was then cooled in an ice bath to repress hydrolysis, and an equal volume of hexane and 3 ml of distilled water were added. The acetonides were recovered from the hexane phase after it had been dried over sodium sulfate. For further analyses the compounds were purified on TLC or by silicic acid column chromatography with benzene or benzene–hexane 1:1, respectively, as eluent. No original diol was observed, which indicated that the reaction was quantitative.

Gas Chromatography

A Barber-Colman Model 10 gas chromatograph with a hydrogen flame ionization detector and a Glowal Chromalab gas chromatograph with an argon ionization detector were used.

Two columns were employed for the GLC of the fatty acids: Column A—2 m × 3 mm I.D., 3% SE-30 siloxane polymer (General Electric) on 100–140 mesh Gas-Chrom P (Applied Science Laboratories, Inc., State College, Pa.) at 150°C; and column B—4 m × 3 mm I. D., 3% EGSS–X (ethylene glycol succinate polyester combined with a silicone) on Gas-Chrom P at 160°C.

For the uropygiols and their derivatives column A was used as above, but at 205°C. In addition a column

4 m × 4 mm, 4% SE-30 on Gas-Chrom P at 260°C, was employed.

Preparative GLC of Individual Uropygiol Acetonides

The column was a 2.5 m × 12 mm I. D. glass tube filled with 1% SE-30 on Gas-Chrom P. The column exit was fitted with a capillary T-piece that provided a split ratio of about 1:10 between the flame detector and a manually operated fraction collector (Packard Instrument Co., Inc.). Fractions were collected in capsules of 40–100 mesh silicic acid (Mallinckrodt) with glass wool in both ends. In order to obtain pure fractions, we collected only the center portion of each peak in 20 successive runs, each sample injection consisting of about 1 mg of purified acetonides in 20 μl of hexane. After collection the capsules were fitted on a funnel and each fraction was eluted with about 5 ml of benzene.

Mass Spectrometry

An Associated Electrical Industries MS-9 double-focusing mass spectrometer was employed under the following conditions: 70 ev ionizing voltage, 300 μamp ionizing current, 8 kv accelerating voltage.

The sample (~10 μg) was admitted by probe directly into the ion source, which was maintained at a temperature just sufficient to volatilize the sample (100–200°C).

Peak positions were measured relative to perfluorotributylamine as internal standard, and an accuracy of better than 10 ppm was realized. All formulae shown were within 3 millimass units of the calculated value.

RESULTS AND DISCUSSION

The IR spectrum of the unsaponifiable fraction is shown in Fig. 2 for identification purposes. Hydroxyl absorption is evident as a broad band between 3600 and 2630 cm^{-1}. In carbon tetrachloride two bands are observed, one at 3640 cm^{-1} and one at 3608 cm^{-1}; they indicate hydrogen bonding (4). The characteristic doublet at 1400–1360 cm^{-1} due to isopropyl groups (5) is absent.

A gas chromatogram of the product (Fig. 3) shows that it contains three major fractions which belong to a homologous series (see insert, Fig. 3). Comparison with a series of aliphatic C_{16}–C_{20} hydrocarbons proved that the fractions differ by one methylene group.

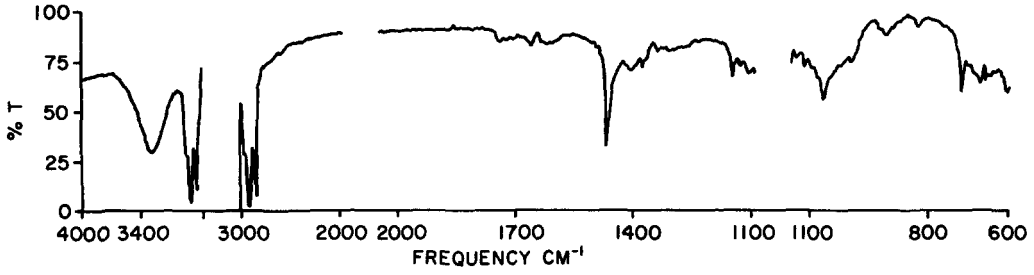


FIG. 2. IR spectrum (KBr) of the purified uropygiols.

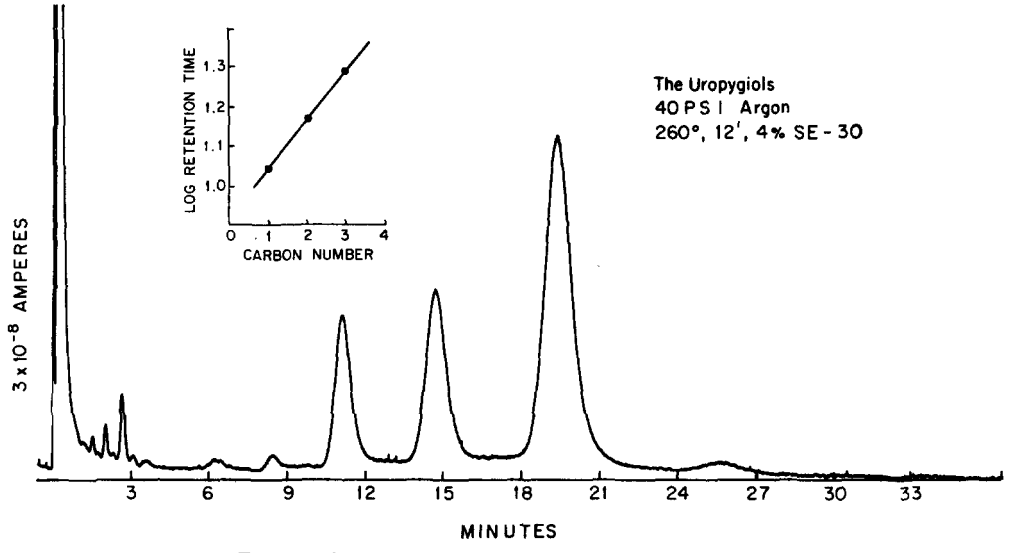


FIG. 3. Gas chromatogram of the purified uropygiols.

Conversion of the product to its acetate results in an increase in retention time by a factor of 1.43 on the nonpolar SE-30 phase. This is in accord with conversion of a diol to a diacetate (methyl 9,10-dihydroxystearate diacetate/methyl 9,10-dihydroxystearate = 1.42) but is not sufficiently different from the corresponding ratio for a monohydroxylic alcohol (octadecyl acetate/octadecanol = 1.52) to characterize the uropygiols as diols. Mass spectral information (see below) suggests an α-glycol linkage, however, and cleavage of the product in tetrahydrofuran with periodic acid yields a mixture of three straight chain aldehydes corresponding in intensity to the original three diols and identified by GLC as n-$CH_3(CH_2)_{18-20}CHO$.

Since 1,2-alkanediols are known to exist in nature (6–8), a model compound, 1,2-nonadecanediol, was prepared by the reaction of osmium tetroxide with 1-nonadecene (95% pure by GLC). The product strongly resembles the uropygiols although some differences exist in its mass spectra that are not explained by its shorter chain length (see below). This model substance also undergoes periodate cleavage—to a C_{18} aldehyde—but its retention time relative to that of this aldehyde is 4.82 while the uropygiols show a corresponding relationship of 3.49.

Reconsideration of the mass spectrum (see below) then led us to consider the presence of a 2,3-diol function.

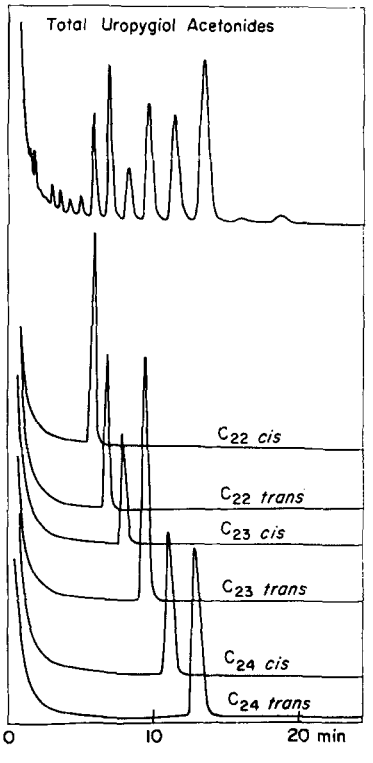


FIG. 4. GLC of uropygiol acetonides: total sample and isolated fractions (preparative GLC). Column A: 3% SE-30 at 130°C.

TABLE 1 RETENTION TIMES OF ACETONIDES RELATIVE TO THOSE OF THE CORRESPONDING DIOLS (4% SE-30, 240°C)

Compound	*Cis*	*Trans*	1,2-Diol
C_{22} Uropygiols	0.85	0.76	
C_{23} "	0.90	0.78	
C_{24} "	0.92	0.79	
2,3-Nonadecanediols	0.90	0.77	
Methyl *threo*-9,10-dihydroxystearate		0.69	
1,2-Nonadecanediol			0.89
Batyl alcohol			0.98

The analogous 2,3-nonadecanediol was prepared by oxidation of a sample of *cis*- and *trans*-2,3-nonadecene, purified by preparative GLC to remove most of the 1,2-isomer. This product, necessarily a mixture of *threo* and *erythro* isomers, mp 75–83°C (compare the uropygiols, mp 73–85°C), shows IR and mass spectra nearly identical with those of the uropygiols. On the nonpolar SE-30 phase only one GLC peak is observed and upon periodate cleavage the expected C_{17} aldehyde is obtained through the loss of two carbon atoms. The retention time of the diol relative to that of its cleavage aldehyde is 3.46, nearly identical with the ratio obtained from the uropygiols.[1]

The uropygiols, now represented by the formula $CH_3(CH_2)_{18-20}$—CHOH—CHOH—CH_3, present a stereochemical problem not encountered in the 1,2-diols. *Threo* and *erythro* diols would not be expected to exhibit any marked mass spectral differences and their gas chromatographic separation factor on liquid phases such as SE-30 might be expected to be small. On the other hand, acetonide derivatives[2] are rigid structures; the *threo* isomer is converted to a *trans* substituted ring while the *erythro* isomer is converted to a *cis* substituted ring. Any geometric differences are thus maximized. When the uropygiols are quantitatively converted to their acetonides and analyzed by GLC, the initial three peaks are replaced by six major peaks with shorter retention times (Fig. 4). Comparison with acetonides of known diols (Table 1) suggests that about equal amounts of *threo* and *erythro* diols were originally present. Fig. 4 shows chromatograms of these acetonides after isolation by preparative GLC. By analogy with the behavior of *cis*- and *trans*-olefins (9) it is assumed that the *cis*-acetonide precedes the *trans*-acetonide in a nonpolar GLC separation (SE-30); this is the basis of the designations in Table 1.

[1] This type of relationship may prove to be one of the simplest methods for locating double bonds within a chain whenever glycol formation by the action of OsO_4 is possible, since it is sensitive to the size of both fragments.

[2] Acetonides have recently been recommended as suitable derivatives for the characterization of unsaturated fatty acid esters by GLC (Horning, E. C., personal communication; see also reference 14).

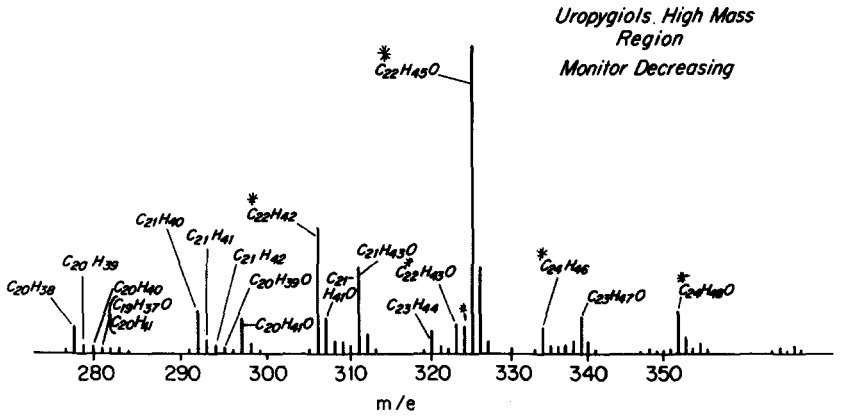


FIG. 5. Upper mass region for the purified uropygiols, showing formulae derived from accurate mass measurements. Starred peaks diminished simultaneously as evaporation proceeded.

The known fatty glycols from wool wax, e.g., 20-methylheneicosane-1,2-diol (6, 7) and other methylated glycols (8), always seem to be 1,2- rather than 2,3-diols. Since the 1,2-diols are configurationally related to corresponding α-hydroxy fatty acids (10), they probably arise by a different process from the one that gives rise to the uropygiols. At present, no other data appear to be available on naturally occurring 2,3-diols.

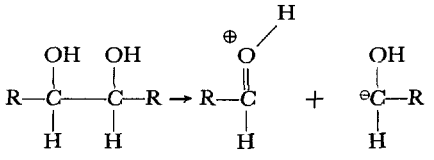
The original lipid, the uropygiol diester, thus belongs to the diester wax group of lipids that recently has been found in sebum-like material of various animals (3, 11).

Mass Spectra

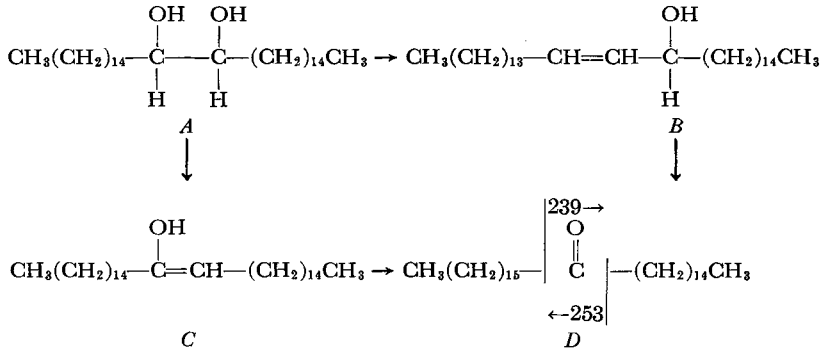
The low-resolution mass spectrum of the purified uropygiols was only partially informative, since the many odd m/e values observed at higher masses indicated that parent ions were not being observed. The small number of discontinuities in the progression of peaks towards lower m/e values suggests the absence of extensive branching. Lowering the ionizing voltage to 10 ev caused a nearly simultaneous diminution of both even and odd mass peaks, which confirmed that the even mass ions are not parent ions arising from pyrolysis in the ion source or inlet, but are being formed during or after the electronic ionization process. The source temperature of the mass spectrometer was only 150°C and pyrolysis would be expected to be small in any case.

Since the material was known from gas chromatography to be a mixture, repeated scans of the high mass region were performed as successively less volatile components evaporated from the probe. The ions starred in Fig. 5 were observed to rise and fall in intensity simultaneously and are therefore presumed to arise from one of the components of the mixture. Mass measurements were then performed relative to perfluorotributylamine as internal standard at a resolution of 10,000 (M/ΔM, 10% valley) on the high mass region, and the ions were assigned the formulas indicated in Fig. 5. The formulas show that none of the ions contains more than one oxygen atom. The ions containing oxygen are observed either to be fully saturated fragments less one hydrogen atom or to contain one point of unsaturation, presumably a double bond. The nonoxygenated fragments, on the other hand, are never saturated, but contain from one to three points of unsaturation, presumably also double bonds.

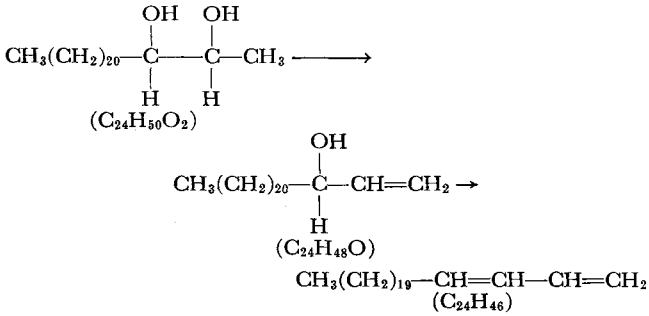
Usually, saturated oxygenated hydrocarbons (esters, ketones, ethers, etc.) show fairly well-defined parent ions, but α,β-diols are an exception. Thus, 1,2-butanediol shows a very small parent ion (12), presumably due to the process:



Ahlquist, Ryhage, Stenhagen, and von Sydow (13), in connection with studies on phthiocerol, obtained the mass spectrum of 16,17-dihydroxydotriacontane (*A*, below), but ions corresponding to simple cleavage at the diol group were absent. Instead, ions corresponding to cleavage with loss of two hydrogen atoms are observed. It is difficult to reconcile the formation of these ions with the postulated original structure, even if one allows for dehydration in the inlet system, a possibility they considered. For example, the prominent ion at m/e 239 corresponding to $C_{16}H_{31}O$ (or, less likely, $C_{17}H_{35}$) cannot logically be derived directly from the expected dehydration product *B*. However, dehydration in the other direction to give *C*, and ketonization of the enol to *D* explain the ions at both m/e 239 and 253. Since the recorded scan did not extend to the expected parent ion at m/e 482, support for this hypothesis is not available.



In the uropygiols, tested under much milder conditions, the intense ion of formula $C_{22}H_{45}O$ (Fig. 5) suggested that the partial structure CH_3-$(CH_2)_{20}$-$CHOH^+$ was derived from an α,β-diol. Since the ions of formula $C_{24}H_{48}O$ and $C_{24}H_{46}$ are derived from the same molecular species (starred ions, Fig. 5), the remaining part of the chain may consist of two carbons and one oxygen atom. These ions would then correspond to the loss of one and two molecules of water respectively from the nonexistent parent ion.



The α-fission fragment $C_{22}H_{45}O$ can lose either two or one hydrogens to form secondary fragment ions at m/e 323 and 324. These ions then lose OH and HOH, respectively, to yield the diene ion at m/e 306 ($C_{22}H_{42}$). Bands corresponding to metastable ions for both processes (loss of OH and HOH) are visible at m/e 289.3 and m/e 290.2.

The spectrum of the *threo* and *erythro* mixture of 2,3-nonadecanediol (Fig. 6) was nearly identical with that of the uropygiols, if allowance is made for the difference of five methylene units. Pertinent ions were accurately measured to confirm their composition. The 1,2-nonadecanediol isomer (Fig. 7), on the other hand, exhibited an intense ion ($C_{18}H_{37}O$) one methylene unit higher in mass than the corresponding ion of the 2,3-isomer ($C_{17}H_{35}O$).

These highly diagnostic ions supply unequivocal proof for the location of the glycol function and, provided glycol formation can be brought about, this may be the best method available for locating an olefin in a straight chain by mass spectroscopy.

Since the acetonides of such glycols have recently been recommended by McCloskey and McClelland (14) for similar purposes, we have investigated the spectra of the *cis*- and *trans*-uropygiol acetonides. The records obtained for the two isomers of the C_{24} diol were very similar; they exhibited a major peak at P − 15 as described by the above authors (14). The differences between the homologues were considerably less well defined than in the diols themselves, and the main advantage of the acetonides appears to lie in their increased volatility and in determination of molecular weight through the P − 15 ion.

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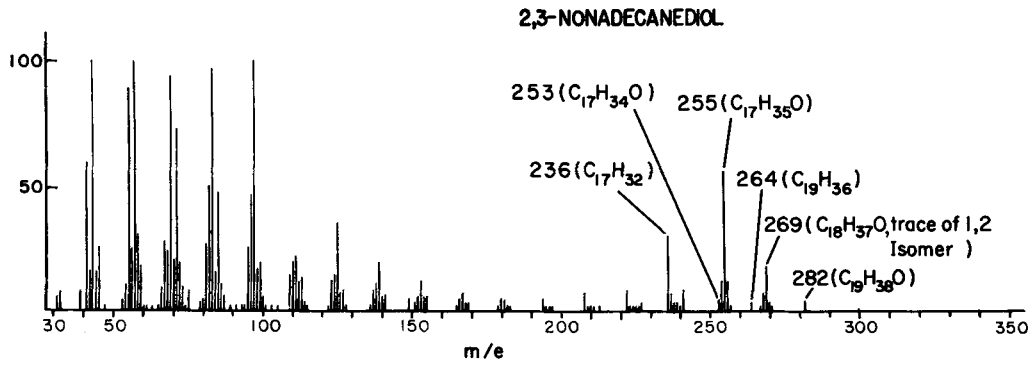

FIG. 6. Mass spectrum of 2,3-nonadecanediol.

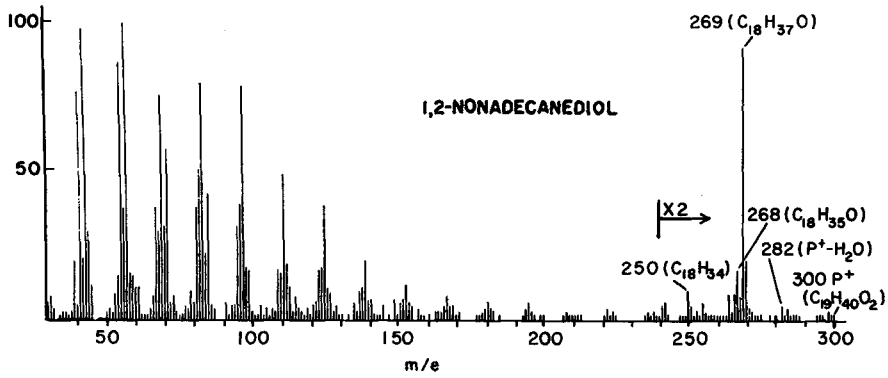

FIG. 7. Mass spectrum of 1,2-nonadecanediol.

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